

### Diagnosis and detection of swine zoonotic diseases and pork hazards

#### **A Multiplex PCR Assay for Detection of *Salmonella* sp., *Listeria monocytogenes*, *Campylobacter* sp., *Staphylococcus aureus* and *Escherichia coli***

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##### **Abstract**

Contaminated food and water are usual vehicles for bacterial pathogens transmission. According to EFSA they promote foodborne illness. *Salmonella* sp., *Campylobacter* sp., *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus aureus* are the most foodborne pathogens reported. Bacterial contamination levels remain at high level, particularly in Europe, despite regulatory efforts to address the situation. The need of new diagnostic tools is crucial. Ideally, tests easy to perform, enough accurate and low cost. The present work talk about optimization of a multiplex PCR (mPCR) test used to detect 5 foodborne contaminants: *Salmonella* sp., *Campylobacter* sp., *L. monocytogenes*, *E. coli* and *S. aureus*. For specificity evaluation, 5 PCR amplification reference DNA were used respectively: 103bp, 174bp, 151bp, 121bp and 136bp. No amplification was observed when primers and DNA from mismatching species were subject to PCR amplification. Furthermore, the sensitivity of this assay was evaluated by using serial dilutions of DNA extracted from clean 1CFU culture of each pathogen. This assay will be optimized by using Real-Time PCR and DNA plasmids containing a single copy of each gene, towards a new and rapid test for food and food manipulated surfaces control. Results are promising and allow us to postulate the design of an accurate and useful assay for bacterial control.

##### **Introduction**

Scientific literature indicates more than 1415 species known to be pathogenic for humans, and 61% of them are zoonotic (Taylor, et al., 2001). Despite the efforts done by industries, foodborne pathogens continue to be a challenge to public health institutions and a threat for consumers (Garrido, et al., 2013). According EFSA data, human cases of infections by *Campylobacter* sp. has shown a slightly decreased in 2012 for the first time in five years, but remain responsible for 214000 infections (EFSA 2014). *Salmonella* sp. is recognized as a major human foodborne pathogen (CDC 2008) and represents a human health concern (Carraco, et al., 2012). Human infection by *Salmonella* sp. has been decreasing, even if 91034 cases have been reported in 2012 (EFSA 2014). Human infections by *Listeria* sp., mainly by *L. monocytogenes* accounted for 10,5% more reported cases in 2012 than in 2011 and has been gradually increasing over the past five years (EFSA 2014). *E. coli* is a common commensal bacterium of mammals. However, several strains integrate virulence factors promoting diarrhea, urologic, or systemic illnesses (CDC 2012, Jandhyala, et al., 2013). *S. aureus* is commonly associated with staphylococcal food poisoning (Alarcón et al., 2006). All these bacteria cause serious problem for human and animal health. So, it is of utmost importance to detect them by tracing food chain with rapid, sensitive, specific and low cost diagnostic tests (Fisher, et al., 2007). The aim of this work is to perform a new mPCR assay to detect these five foodborne pathogens.

## Material and Methods

### Primer design

The sequences of the oligonucleotide primers used in this study were designed based on specific target genes present in each species. The primers were designed to have the same annealing temperature, and the targets were chosen based on recent publications and the most specific and reliable genetic targets for the five pathogens (Table 1). Primers were designed using the Primer Express 2.0.0 Software program and synthesized by NZYTech Company (Portugal). The primers sequences will not be described due to safeguard of intellectual property issues.

Table 1

Reference strain, target gene and lyophilized pellets used in the specific detection of *Salmonella* sp., *E. coli*, *L. monocytogenes*, *Campylobacter* sp. and *S. aureus* and amplicon size.

Food borne pathogen	Target gene	Reference strains	Tablets	Amplicon size (bp)
<i>Salmonella</i> sp.	gapA	<i>S. enterica</i> serotip Budapest (INSA)	NCTC 6017	103
<i>Escherichia coli</i>	uspA	CECT 105	ATCC 25922	121
<i>Listeria monocytogenes</i>	hlyA	CECT 934	ATCC 13932	151
<i>Campylobacter</i> sp.	cdtB	CECT 7572	ATCC 33291	174
<i>Staphylococcus aureus</i>	16S RNA	CECT 239	ATCC 25923	136

### Sensitivity and specificity

The specificity of this assay was evaluated in two phases: i) by using CECT reference DNA's, and ii) by using reference tablets of reference strains. Tablets were dissolved in 20mL of sterile water by 10', and plated on agar medium for 18h, in accordance with the manufacturer's instructions. In the case of *C. jejuni*, this strain was plated on agar-blood medium. After 18h of growth, it was selected one CFU and all DNA's were extracted with QIAamp® DNA Mini and Blood Mini Handbook kit (QIAGEN). Concerning *Salmonella* sp., we have utilized a reference strain confirmed by the Portuguese Reference Laboratory (INSA Instituto Nacional de Saúde Dr Ricardo Jorge).

The specificity of the designed primers was tested using Blast®, NCBI Software on-line program and was confirmed by PCR. The sensitivity of this assay was tested by doing different 10x dilutions, starting from the genomic DNA extracted by 1CFU provided by the reference strains. Then, the PCR was performed until no amplification was observed in agarose gel. Finally, all DNA was quantified by using NanoDrop™ and Quant-iT™ PicoGreen® to determine the reaction sensitivity.

### PCR conditions

Amplification of bacterial DNA was performed by using 10µL MyTaq™RedMix2X (Bioline), 300µM of primer mix and 1µL of DNA template, in a 20µL reaction. The PCR was performed under the following conditions: 5' at 95°C; 40 cycles of 20'' at 95°C, 20'' at 56°C, 20'' at 72°C; and a final extension step of 5' at 72°C. PCR products were analyzed by agarose gel electrophoresis. 10µL of the PCR products were loaded into 3% agarose gel and subjected to electrophoresis for 45' at 120V in TAE buffer. Gels were observed and documented using the Gel-Doc UV (Bio-Rad).

## Results

### *Specificity and sensitivity of the assay*

The tests were performed in order to evaluate the specificity of the mPCR reactions. Results are shown in Figure 1, Figure 2 and Figure 3. Briefly; specificity tests were made on two phases: i) a PCR mix containing 5 pairs of primers was used to amplify DNA from each specie; ii) a PCR mix containing all pairs of primers and a mixture of DNA provided from the 5 species. The DNA used was obtained from tablets from each species. The PCR mix containing all pairs of primers and DNA from each species to prevent cross reaction of primers between the species i.e., the ability of a certain pair of primers to amplify a target from a different species. No cross reaction was observed for each set of amplification (Figure 1). The mPCR mix containing all pairs of primer and DNA from the 5 species, allowed us to evaluate the ability to detect all species present in each sample. All except *S. aureus* were amplified (Figure 2). The quality or quantity of DNA utilized is critical (data not shown). Our results suggest that amplification of *S. aureus* DNA is more performing in a single reaction together with *Campylobacter* sp. Remaining pathogens amplifications (*E. coli*, *L. monocytogenes* and *Salmonella* sp.) works perfectly all together, even in the presence of all 5 pairs of primers. The size of amplicons corresponded to the expected size and no additional or nonspecific bands were observed in the PCR reactions (Figure 3).

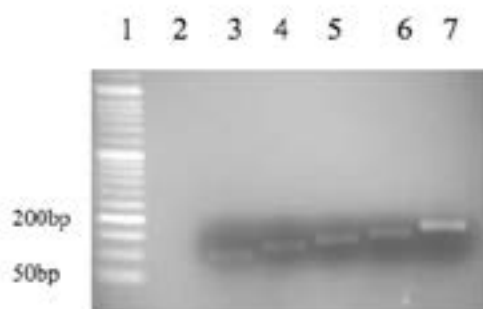


Figure 1

The DNA agarose gel (3%) demonstrating the PCR mix containing all pairs of primers and only one DNA from each species: lane 1 – DNA marker, lane 2 – negative control, lane 3 – *Salmonella* sp., lane 4 – *E. coli*, lane 5 – *S. aureus*, lane 6 – *L. monocytogenes*, lane 7 – *Campylobacter* sp.

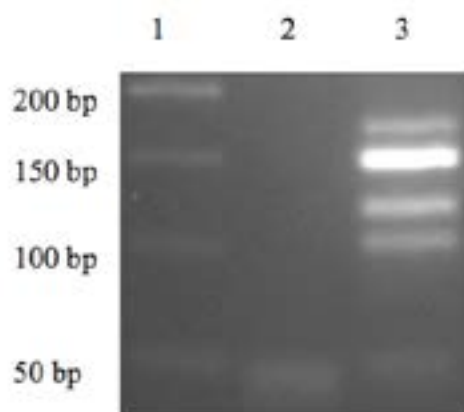


Figure 2

The DNA agarose ge (3%) demonstrating mPCR samples for amplification of the genomic DNA provided by CECT and INSA: lane 1 – DNA marker, lane 2 – Negative control, lane 3 – amplification of *L. monocytogenes*, *Campylobacter* sp., *E. coli* and *Salmonella* sp.



Figure 3

The DNA agarose gel (3%) demonstrating PCR samples for amplification of the genomic DNA provided by tablets: lane 1 – DNA marker, lane 2 demonstrating PCR samples for amplification of the – negative control, lane 3 – amplification of *Campylobacter* sp. and *S. aureus*, lane 4 – amplification of *L. monocytogenes*, *E. coli* and *Salmonella* sp.

A limit dilution approach was made in order to evaluate the optimal concentration of DNA for amplification reaction. Briefly, DNA corresponding to one CFU from the reference strains was used to prepare 10x dilutions, and PCR amplification. DNA corresponding to the last dilution with amplification was determined for each pathogen. DNA was quantified by using NanoDrop™ and Quant-iT™ PicoGreen®. The working dilution was achieved at 11 dilutions to *Salmonella* sp., 5 to *E. coli*, 2 to *L. monocytogenes*, 5 to *Campylobacter* sp. and 5 to *S. aureus*.

## Discussion

The accuracy of mPCR was evaluated at different levels. Initially, we have used DNA of each reference strain with a mix containing all the 5 pairs of primers. Any nonspecific band was observed (Figure 1). In the second step, DNA from all bacteria was mixed in the same reaction mix. As shown in Figure 2, *S. aureus* gene was not amplified. As referred previously, several experiments were performed to improve mPCR. In this attempt, several reaction conditions were performed, namely the number of extension cycles, primers and DNA concentration, primer melting temperatures etc. (data not shown). Results were not satisfactory in previously defined conditions. So, a combination of *S. aureus* DNA and *Campylobacter* sp. DNA was proposed in a single reaction with a mix of 5 pair of primers; in a separated set, *E. coli*, *L. monocytogenes* and *Salmonella* sp. DNA was amplified with the mix of 5 pairs of primers. In this condition, multiplex PCR amplification was successful. Regarding the sensitivity, a limit dilution approach was used, as referred before. Starting from DNA extracted from 1CFU, we performed several dilutions and submitted these to PCR. We observed a decrease in the amount of PCR product obtained with the increase in dilutions. The limit was obtained when a further dilution did not generate any PCR product. Then, DNA from each pathogen was quantified by using NanoDrop™ and Quant-iT™ PicoGreen®. Surprisingly, it was difficult to quantify the amount of DNA even in the non-diluted samples with either technique. For this reason it was not possible to determine the amount of DNA present in the dilutions to infer the sensitivity of each mPCR. This may be explained by the sensitivity of NanoDrop™ apparatus (up to 2ng/μl), and the Quant-iT™ PicoGreen® approach, which ranges from 50 pg to 2 μg. The PCR sensitivity, depending on the target and other factors, reaches lower values, but this is not true for the quantification techniques used. These facts justify the positive PCR amplification from all the samples even if the DNA quantification didn't work. Nevertheless, the authors started a plasmid based approach to evaluate with higher accuracy the sensitivity of the mPCR reactions. Briefly, the 5 genes were independently cloned or inserted in a bacterial plasmid (pGEM®-T Easy) and used to transform *E. coli* cells. The positive clones were confirmed by restriction enzyme analysis and were sent for sequencing in an external laboratory. After confirming the identity of the plasmids containing the genes, high quantities of plasmid DNA will be produced and purified, which will be easily quantified by the described methods, and then used in the same limit dilution approach used to evaluate the sensitivity of each reaction. This work is under development and due to that, data is not available.

## Conclusion

PCR amplification has been widely accepted as the method of choice for rapid and reliable detection of zoonotic agents in food. However, this detection is not yet used in food microbiological analysis probably because there is still a lack of standardized criteria for validation of PCR sample preparation methods, reaction components and assembly, as well as amplification conditions for pathogens and various food matrices (Malorny, et al., 2003; McKillip & Drake, 2004). Considering the reaction time, specificity and detection limit defined, it can be inferred that the mPCR proposed, can be used as first step in studies

for detection of foodborne pathogens. The present study illustrates a new diagnostic test that enables the simultaneous detection of 5 important foodborne pathogens. Currently the test identifies all of the 5 bacteria, and the next steps will be to adapt it to the needs of the food diagnostic market.

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